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Award Number: DAMD17-02-1-0254

TITLE: Prostate Activated Prodrugs and Imaging Agents

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REPORT DATE: May 2003

TYPE OF REPORT: Annual

20060223 077

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-05-2003		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 May 2002 - 30 Apr 2003	
4. TITLE AND SUBTITLE Prostate Activated Prodrugs and Imaging Agents				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER DAMD17-02-1-0254	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Graham B. Jones, Ph.D. E-Mail: gr.jones@neu.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Northeastern University Boston, Massachusetts 02115-5000				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited.					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this project is to demonstrate that enzymatically active prostate specific antigen (PSA) in the prostatic microenvironment can be used to locally activate either prodrugs or imaging systems. The substrate chosen was a 3 component system composed of a peptide sequence with affinity for PSA, an imaging agent / cytotoxin and a deactivating bridge-linker, which electronically incapacitates the imaging agent. On PSA mediated release, the peptide sequence is designed to uncouple from the bridge, which then undergoes spontaneous decomposition, releasing free imaging agent or cytotoxin. The linker selected was 4-amino benzyl alcohol and a model substrate [tyrosine] was coupled to the linker and attachment to the clinically used prostatic cancer agent doxorubicin and various imaging agents attempted. Despite numerous attempts, hampered by a combination of chemical instability and incompatible functionality, preparation of desired conjugates was impeded. However, the limiting factors preventing chemical synthesis were uncovered, and the syntheses re-engineered, allowing formation of the first substrate for biological evaluation.					
15. SUBJECT TERMS Chemical synthesis, prodrug, targeted chemotherapy, imaging agents, prostate specific antigen					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 10	19a. NAME OF RESPONSIBLE PERSON
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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Introduction:

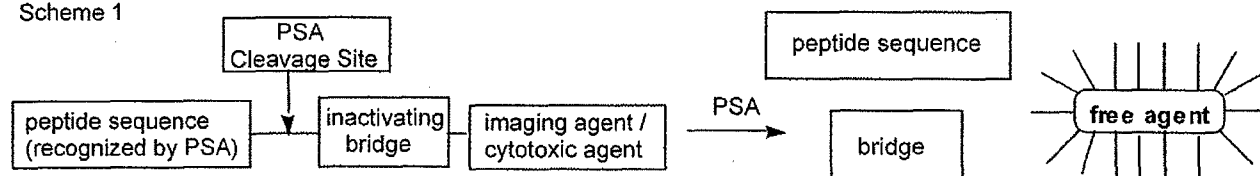
The overall goal of this project is to demonstrate that enzymatically active PSA in the prostatic microenvironment can be used to locally activate prodrugs and imaging systems. The research is based on the finding that PSA is enzymatically active, and has a restricted pattern of peptide bond cleavage. To demonstrate this we have designed substrates for PSA that have 3 components: (1) a peptide linkage with affinity for PSA (2) a latent image contrast agent / cytotoxic prodrug and (3) a deactivating bridge, which electronically incapacitates the imaging agent until PSA activates the substrate.

Body: As outlined in the Statement of Work, the initial research plan called for design of the three component PSA substrates (objective 1), chemical synthesis of the agents (objective 2), investigation of enzymatic activation (objective 3) and subsequent biological studies and refinement (objectives 4-6). In the initial reporting period we have concentrated effort on reducing to practice the appropriate synthetic chemistry protocols required to enable us to prepare large libraries of compounds for biological evaluation. A number of unanticipated hurdles were encountered during the initial phases of the work, and solutions to overcome the problems were developed.

Rationale for agent design

Although metastatic prostate cancer typically responds to hormonal therapy in the form of androgen ablation, this therapy is time limited,¹ and systemic therapies for the androgen independent phase of the illness are needed. Of significance, prostate cancer, even after multiple hormonal and chemotherapeutic manipulations, typically continues to secrete prostate specific antigen (PSA) during relapse. Therefore, the development of an agent that could be selectively activated by PSA either within prostate cells or their microenvironment is of great interest. PSA is known to have a protease activity with a relatively restricted pattern of peptide cleavage,² and continues to be a relevant tumor marker in well over 90% of late stage patients.³ In view of this, PSA activated prodrugs constitute a potentially attractive proposition. Our rationale was to attach a PSA substrate to a cytotoxin which is rendered inert via conjugation through an insulating chemical bridging unit (Scheme 1). On PSA mediated release, the bridge undergoes spontaneous decomposition releasing the activated cytotoxin. If designed carefully, such a strategy might also apply to the PSA mediated release of imaging agents, whose fluorescent capacity on release would serve as a chemical

Scheme 1



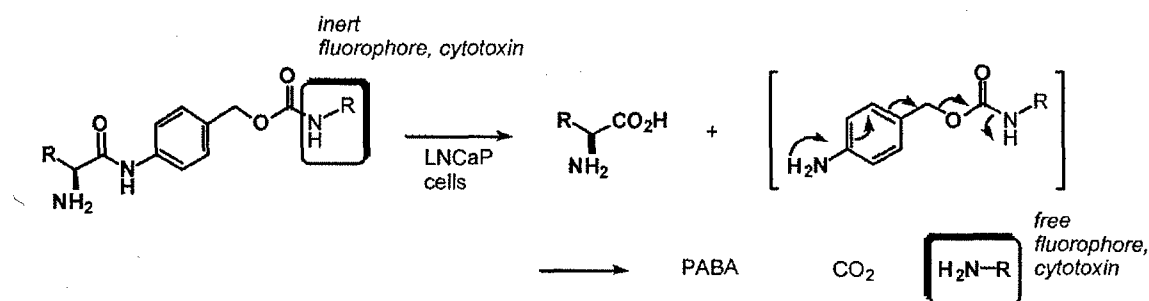
sensor of PSA activity, thus prostate cancer progression.

Choice of peptide sequence

A number of PSA activated substrates have been reported, including the HSSKLQ hexapeptide.⁴ Our desire, in proof of principle studies was to employ a minimal substrate and selected tyrosine (Y) conjugates for development based on their ease of derivitization. Such a strategy would allow us to optimize the coupling chemistry required to attach the insulating linker and the designated cytotoxins & imaging agents. In the case of Y conjugates, in addition to PSA mediated release we would also be in a position to evaluate competing activation by α -chymotrypsin, and then assemble chimeras where specificity for PSA was achieved. Concurrent with this work, we would assemble synthetic derivatives of the HSSKL motif for eventual coupling.

Choice of bridging linker

Our preferred choice for the inert linker is the *p*-aminobenzyl alcohol pioneered by Katzenellenbogen.⁵ This allows coupling of peptide based enzyme substrates through the N terminus, with the alcohol group incorporated into a carbamate which masks the amino containing molecule targeted for delivery. Selective enzymatic hydrolysis of the amide group results in (a) generation an exomethylene iminium ion which is then captured by water to regenerate the free linker (b) concomitant expulsion of CO₂, rendering the reactions essentially irreversible and (c) expulsion of the free amine as shown in Scheme 2. The key is to harness amino containing substrates in the system where differences in the chemistry between the carbamate and amino form are pronounced. Amine containing cytotoxins where the basicity of the amino group plays a role in activity were thus sought, and adriamycin (doxorubicin) selected for initial development. In the case of



Scheme 2. PSA mediated release of Tyr-linker conjugates

imaging agents, classical anilino containing fluorophores were deemed key substrates for this system, as discernable differences in UV and fluorescence characteristics would be expected for the free aniline as opposed to the linked carbamate form.

Choice of cytotoxin

We initially selected doxorubicin (adriamycin) for incorporation into the 3 component PSA activated system. Adriamycin is one of very few agents that has been shown to be active against prostate cancers.⁶ Additionally, an analog of adriamycin, mitoxantrone is the first chemotherapeutic agent to have an FDA indicated usage for therapy of metastatic prostate cancer.⁷ Of key importance, the amine group on adriamycin makes a convenient site for coupling to the peptide / linker. The mechanism by which adriamycin functions as an antitumor agent is reasoned to involve association with DNA in such a manner that the anthraquinone unit stacks (intercalates) between the base pairs, and the aminosugar group points outwards to the minor groove of the DNA.⁸ DNA cleavage is then mediated through involvement of the regulatory enzyme topoisomerase II. A key feature of interactions of the aminosugar with DNA relies on the fact that the basic amino group can be protonated, and that the resulting ammonium ion participates in interactions with the minor groove and possibly the sugar-phosphate backbone of DNA. These interactions are possible in the case of free adriamycin, but, importantly, far less likely when the aminosugar group of adriamycin is coupled to either a linker group or peptide which introduces severe steric burden and, more importantly, can prevent the nitrogen atom from being charged and participating in attractive ionic interactions with DNA. By inactivating adriamycin via an amide type bond to the linker, the basicity of the nitrogen atom of the aminosugar is significantly reduced, ultimately preventing efficient association with DNA. Simple N-alkyl derivatives of the agent still associate with DNA and can cause topoisomerase mediated strand breaks, but N-acyl analogs show negligible association to DNA, and instead operate by other mechanisms. Thus, utilizing adriamycin in a 'deactivating' acyl type bridge (carbamate) between the linker and its aminosugar group constitutes a rational strategy.

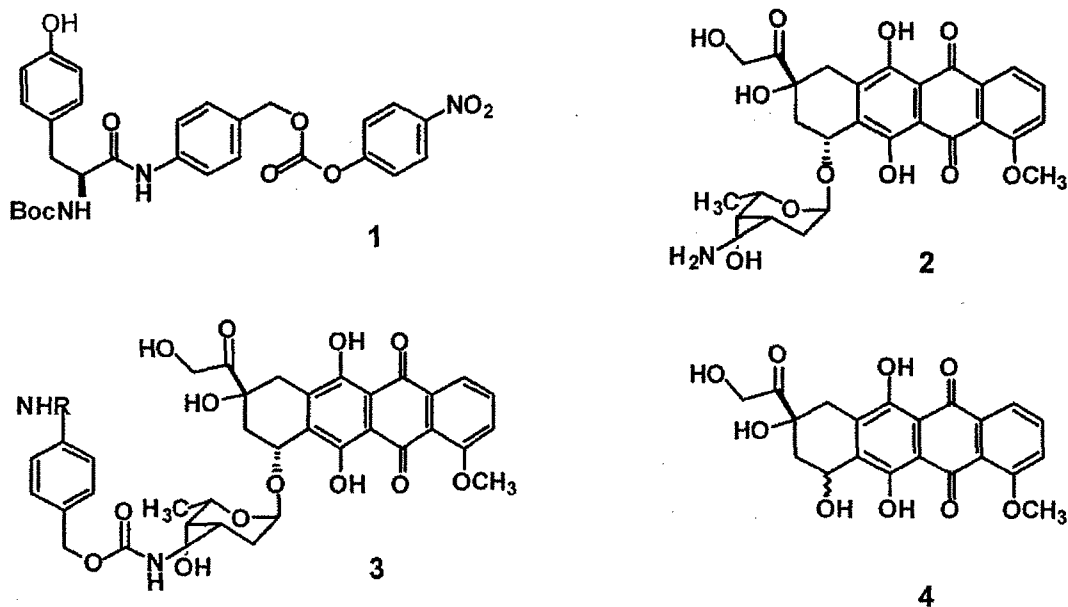
Choice of fluorophores

A number of image contrast enhancing agents have been studied for use in prostate cancer in conjunction with ultrasound methods of detection with varying degrees of success.⁹ An attractive possibility lies in the design of an imaging system, which exploits the enzymatic efficiency of PSA in the prostatic microenvironment. Our strategy was to conjugate a proteinogenic PSA substrate to a masked aniline type fluorophore via the inert spacer/linker group, such that the free amine fluorescent molecule is liberated on proteolysis (Scheme 1).¹⁰ Our goal was to initially investigate coupling with readily available aniline based fluorophore dyes. Rhodamines, luciferins and the Cy dyes were selected for investigation on the basis that their UV characteristics are all greatly influenced by the electron donating capacity of the anilino nitrogen group.

Results of investigation

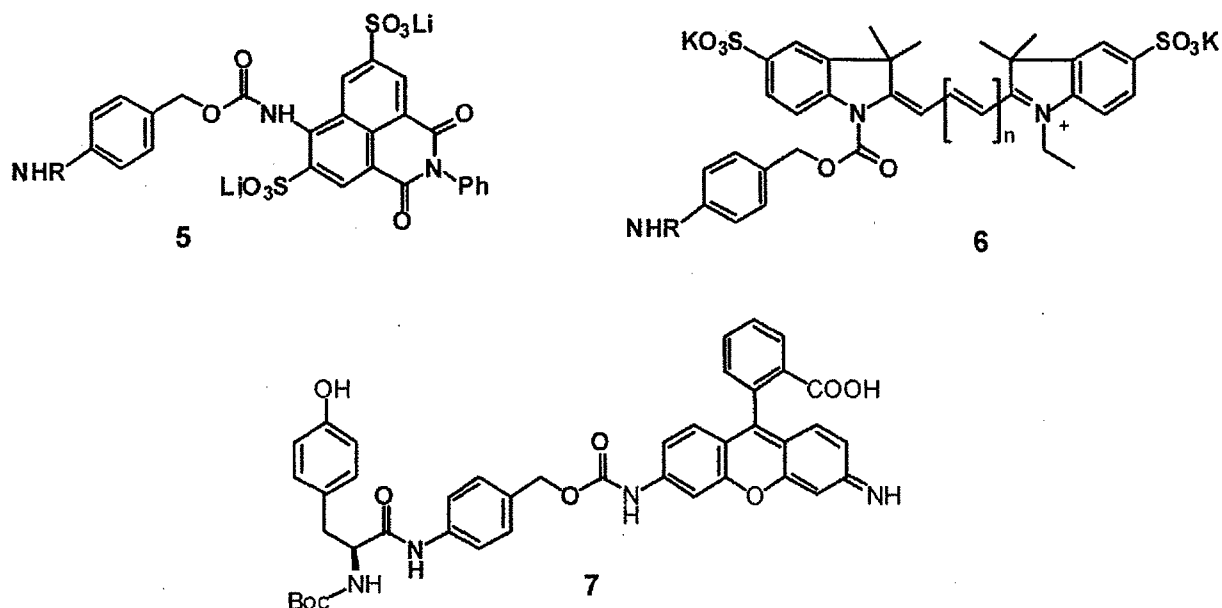
Considerable effort was directed towards synthesis of a common coupling agent which would permit

introduction of various imaging agents and cytotoxins. Substantial quantities of intermediate **1** were prepared, which constitutes a masked version of the peptide substrate coupled to the linker, equipped with activating functionality to allow introduction of key prodrugs. Our initial efforts focussed on attaching adriamycin (**2**) to the template to produce the 3 component conjugate (**3**, R=tyrosyl). Despite considerable effort and attention to numerous coupling parameters, successful assembly of **3** (R=tyrosyl) was not achieved. Various iterations investigated included coupling of the underivatized linker (**3**, R=H), unprotected Y-linker conjugates and intermediate activation of the amino group of **2**. In the majority of cases, the key culprit was chemical instability of the glycosidic linkage of **2**, resulting in formation of des-glycone **4** under the coupling conditions. Based on this finding, the coupling to adriamycin was deferred in favor of more promising proof of principle applications using more chemically robust imaging agents.



With activated coupling agent **1** available, synthesis of a number of fluorophore conjugates were investigated. The initial target studied was luciferin conjugate **5**. However, though a range of coupling strategies were screened, the efficiency of formation and isolation of **5** could not be rendered practically useful. Contributing factors may have included the ion chelating capacity of the sulfonic acids, and the reduced nucleophilic ability of the aniline nitrogen atom due to inductive effects. Similar problems were encountered en route to Cy dye derivative **6** with negligible reactivity of the key nitrogen atom precluding practical synthesis. Compounding the investigation, intractable mixture of products were typically encountered, and despite substantial effort using various chromatographic methods, purified product could not be obtained. Based on these findings, we moved attention to use of alternate aniline fluorophores. Finally, a successful synthesis of the rhodamine conjugate **7** was achieved. The procedures were amenable to large scale production, but a major obstacle was encountered in attempting to remove the Boc protecting group on the tyrosine unit. Traditional conditions led to decomposition of material, with only trace quantities of the desired product attainable. Its successful preparation did however allow us to confirm hypothesis in that the

UV characteristics of the molecule were markedly different to free rhodamine dye. This supported the notion that PSA mediated hydrolysis could be tracked spectrophotometrically. Clearly, remedy was needed to overcome the protecting group hurdle, and molecule 7 was used for a battery of chemical compatibility studies aimed at identifying the most appropriate protecting group for use on the tyrosine nitrogen atom.



From this it was discovered that Pd mediated processes were compatible with substrate 7, inferring that an alternate [alloc] protecting group would be appropriate means to mask the group and allow facile deprotection to give our desired product. Accordingly, this modification became the immediate priority for year 2.

In related studies, we also investigated synthetic and semi-synthetic routes to the desired HSSKL conjugates that we anticipate attaching to the PSA substrates in order to achieve specificity. After numerous approaches were studied, a route was developed commencing with unprotected HSSKL pentapeptide, which is then converted to the bis-Boc (H, K) derivative for subsequent coupling.

Key research accomplishments:

- Large scale preparation of an activated derivative of tyrosine coupled to a self immolative linker
- Successful coupling of tyr-linker to rhodamine to form image contrast agent with differential spectrophotometric fingerprint
- Determination of scope and limitation of adriamycin and selected dyes in coupling reactions
- Identification of optimal protecting group strategy for assembly of tyrosine-bridged conjugates
- Large scale synthesis of HSSKL pentapeptide building block for subsequent use

Reportable outcomes:**Trainees Mentored**

Ahmed El-Shafey [Staff scientist - bioanalytical division, Pacific Northwestern Laboratories]

Ajay Purohit [BMS molecular imaging division]

Curtis Crasto [Staff scientist, Northeastern University]

Duckhee Lee [Research Scientist, Korea National University]

Walter Samaniego [Senior Chemist, Florida]

Jude Mathews [Staff scientist, Northeastern University]

Longfei Xie [current student]

Jane Li [current student]

Degrees Supported

Ahmed El-Shafey PhD 2003

Ajay Purohit PhD 2002

Conclusions:

Chemical synthesis of three component systems comprised of an enzyme substrate, inert linker and fluorophore /imaging agent has been investigated for activation by the prostate cancer biomarker PSA. A number of challenges were encountered during the chemical synthesis phase, and the results obtained have been used to re-guide future effort and focus. An rhodamine dye conjugate of the enzyme substrate has been prepared, and conditions for its isolation determined. Its spectral characteristics support the design philosophy of the program and its activation under the influence of PSA will be investigated in due course.

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